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On 27 April 2007

TOWNSEND and TOWNSEND and CREW LLP

By: Malinda Adajet

PATENT
Attorney Docket No.: 018512-005910US

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

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|---|---|
| In re application of: | Confirmation No. 8561 |
| Timothy James Jegla | Examiner: CHERNYSHEV, Olga N. |
| Application No.: 10/815,297 | Technology Center/Art Unit: 1649 |
| Filed: March 31, 2004 | DECLARATION UNDER 37 C.F.R. §1.132 OF DR. DOUGLAS S. KRAFTE |
| For: KV10.1, A NOVEL VOLTAGE-GATED POTASSIUM CHANNEL FROM HUMAN BRAIN | |
| Customer No.: 20350 | |

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, DOUGLAS S. KRAFTE, being duly warned that willful false statements and the like are punishable by fine or imprisonment or both (18 U.S.C. § 1001), and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my own knowledge are true, and statements made on information or belief are believed to be true and correct.

2. I received my B.Sc. in molecular biology from Vanderbilt University and my Ph.D. in physiology from the University of Rochester. Currently I am the Vice President of Biology at Icagen, Inc. I have been at this position and related positions for approximately eight years. A copy of my curriculum vitae is attached hereto as **Exhibit A.**

3. The invention of the above-referenced patent application provides for the first time a nucleic acid encoding human Kv10.1, a member of the Kv family of voltage-gated potassium channels, and demonstrates the functional expression of this potassium channel subunit. Kv10.1 defines a previously unidentified Kv10 subfamily of potassium channels and is expressed primarily in the central nervous system (CNS) including brain and spinal cord, in the male reproductive organs including prostate and testis, and in ocular tissue.

4. I have read and am familiar with the contents of this patent application. In addition, I have read the Office Action of March 2, 2006, and the Final Office Action of August 23, 2006, received in this application. It is my understanding that the Examiner does not believe that the present invention is supported by a specific, substantial, and credible asserted utility or a well established utility as required by the United States Patent Laws.

5. This declaration is provided to demonstrate that the identification of the coding sequence for Kv10.1, coupled with the demonstration of functional expression, has a specific and substantial utility, which is credible to one of ordinary skill in the art, particularly for the purpose of drug discovery.

6. Several subfamilies of the Kv potassium channel family have previously been identified. These potassium channels are indicated in signal transduction during various biological processes such as neuronal integration, cardiac pacemaking, muscle contraction, hormone secretion, cell volume regulation, lymphocyte differentiation, and cell proliferation. Given this knowledge and the specific expression of Kv10.1 in the CNS, male reproductive organs, and retina, one of ordinary skill in the art would recognize the Kv10 channel as a therapeutic target for treating CNS or vision disorders or for regulating male infertility. In support of this statement, there is a recent publication describing mutations in the Kv10.1 gene

(KCNV2) that are responsible for a specific vision disorder, which is characterized by reduced visual acuity, photoaversion, night blindness, and abnormal color vision (Wu *et al.*, 2006 *Am. J. Hum. Genet.* **79**: 574-579, attached as **Exhibit B**). The identification of human Kv10.1 coding sequence makes it possible to screen for activators and inhibitors of Kv10 potassium channels. Because such activators or inhibitors can be used for treating abnormalities in the relevant tissues (such as epilepsy, impaired vision, and male infertility), the present invention has a specific and real-world use. A further example of targeting potassium channels for therapeutic purpose is KCNQ2. Loss of function mutations of KCNQ2 have been shown to cause a form of epilepsy (Singh *et al.*, 1998 *Nat. Genetics* **18**: 25-29, attached as **Exhibit C**) and the KCNQ2 channels have been targets for drug discovery programs for a number of years (see, *e.g.*, Wickenden *et al.*, 2004 *Expert Opin. Ther. Patents* **14**(4): 1-13, attached as **Exhibit D**).

7. It is well known in the art that once an ion channel has been identified, modulators of this ion channel can be routinely identified based on the coding sequence of the ion channel, functional expression, and a method for activation of the channel. The present application provides nucleic acid sequences encoding human Kv10.1 polypeptides as well as methods for activating a Kv10 potassium channel, one of ordinary skill in the art can thus conduct routine testing to identify activators or inhibitors of a Kv10 potassium channel useful for modulating signal transduction in the cells where this potassium channel is present (*e.g.*, the brain, spinal cord, prostate, testis, and retina), and therefore useful for treating neurological disorders and vision problems, or for modulating male fertility.

8. There are known instances where modulation of an ion channel is useful for treating a specific disease even though the channel itself may not cause the disease. For example, hypertension can be caused by a variety of illnesses such as renal disease and diabetes. Among the treatment strategies for hypertension is the use of drugs such as calcium channel blockers to relax the vasculature. Relaxing the vasculature to reduce blood pressure by blocking a calcium channel is useful and effective, even if the original cause of the hypertension is unrelated to the calcium channel itself. Similarly, it is perfectly reasonable to expect that the targeting of a Kv10 channel, a voltage-gated potassium channel expressed at a high level in the

CNS, ocular tissue, and male reproductive system, is an appropriate strategy for treating disorders in the CNS or vision, or conditions related to male fertility, whether or not such abnormality is directly caused by altered Kv10 activity. Thus, the disclosure of the present application is sufficient to establish the utility of Kv10.1.

9. In the Office Actions of March 2 and August 23, the Examiner apparently takes the position that the sequence information of Kv10.1 alone is insufficient to establish utility. It is respectively submitted, however, that this patent application provides not only sequence information, but also functional expression and tissue distribution for the Kv10.1 ion channel. In my experience, this disclosure provides the vital information necessary for a modern drug discovery effort where one expresses an ion channel of interest and subsequently identifies small molecule modulators of the ion channel in functional assays; the modulators can then be used for treating diseases and conditions relevant to the ion channel. Many of the drug discovery programs I have been associated with over the years have relied on a similar level of information and data.

10. In summary, it is my scientific opinion that one of skill in the art, at the time the application was filed, would recognize the specific and real-world utility of the Kv10.1 encoding nucleic acids of the present invention.

Date: Apr. 18, 2007

By: _____
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# Exhibit A

# Douglas S. Krafte, Ph.D.

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## Professional Experience

ICAgen, Inc.
Research Triangle Park, NC
Vice President Biology — 2002- present
Director of Biology — 1999- 2002

Aurora Biosciences Corporation
San Diego, CA
Group Leader – Cell Biology — 1997-1999

Boehringer Ingelheim Pharmaceuticals, Inc.
Ridgefield, CT
Senior Principal Scientist – Pharmacology — 1994-1997

Sterling Winthrop Pharmaceuticals Research Division
Collegeville, PA
Principal Research Investigator — 1989-1994

## Education

California Institute of Technology
Pasadena, CA
Research Fellow - Molecular Neurobiology — 1986-1988

University of Rochester
Rochester, NY
M.S./Ph.D. Physiology — 1981-1986

Vanderbilt University
Nashville, TN
B.S. Molecular Biology — 1977-1981

## Areas of Scientific/Technical Expertise

Ion channel biology

- Ion channel biology
- Pre-clinical drug discovery
- HTS methodologies
- Electrophysiological methodology
- General molecular biological methods
- ELISA-based cytokine measurements
- Tissue culture/cellular dissociation
- Computer-assisted data acquisition and analysis

## Professional/Academic Activities

- NIH postdoctoral fellowship (1986-1989)
- Del Webb Foundation fellowship (1986)
- NIH predoctoral fellowship (1982-1985)
- Instructor: Ion Channels in Natural and Model Membranes
  Hopkins Marine Station of Stanford University
- Biophysical Society, Society for General Physiologists, Society for Neuroscience, BIO
- Ad hoc reviewer: Journal of General Physiology; American Journal of Physiology; Receptors and Channels; Biochemistry
- Co-author of 29 refereed manuscripts and 7 book chapters

## PUBLICATIONS

refereed manuscripts


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# Exhibit B

# REPORT

# Mutations in the Gene *KCNV2* Encoding a Voltage-Gated Potassium Channel Subunit Cause "Cone Dystrophy with Supernormal Rod Electroretinogram" in Humans

Huimin Wu,* Jill A. Cowing,* Michel Michaelides, Susan E. Wilkie, Glen Jeffery, Sharon A. Jenkins, Viktoria Mester, Alan C. Bird, Anthony G. Robson, Graham E. Holder, Anthony T. Moore, David M. Hunt, and Andrew R. Webster

"Cone dystrophy with supernormal rod electroretinogram (ERG)" is an autosomal recessive disorder that causes lifelong visual loss combined with a supernormal ERG response to a bright flash of light. We have linked the disorder to a 0.98-cM (1.5-Mb) region on chromosome 9p24, flanked by *rs1112534* and *rs1074449*, using homozygosity mapping in one large consanguineous pedigree. Analysis of one gene within this region, *KCNV2*, showed a homozygous nonsense mutation. Mutations were also found in 17 alleles of 10 other unrelated families with the same disorder. In situ hybridization demonstrated *KCNV2* expression in human rod and cone photoreceptors. The precise function of *KCNV2* in human photoreceptors remains to be determined, although this work suggests that mutations might perturb or abrogate $I_{Kx}$, the potassium current within vertebrate photoreceptor inner segments, which has been shown to set their resting potential and voltage response.

The mechanism by which the sensitivity of the visual system is regulated has been a focus of study for many years. "Cone dystrophy with supernormal rod electroretinogram (ERG)" is unusual, in that affected individuals show a delayed and reduced cone and rod ERG response that switches at higher light levels in rods to an exaggerated or supernormal response. Since the rod ERG b-wave is derived predominantly from bipolar cells in the retina, the ERG phenotype suggests a dysfunction affecting the first synapse of photoreception. The identification of the causative gene(s) would be expected, therefore, to provide insights into the control mechanisms that occur during signal transmission to these second-order neurons.

The disorder was first described in 1983,[1] with five subsequent case series.[2–6] It shows an autosomal recessive pattern of inheritance and is characterized by reduced visual acuity, photoaversion, night blindness, and abnormal color vision. At an early age, the retina shows subtle depigmentation at the macula and, later, more obvious areas of atrophy (fig. 1). Electroretinography is characteristic and is required to make a specific diagnosis (fig. 1). All patients in this study underwent a full ophthalmological examination. Blood samples were taken from affected individuals and family members, for DNA extraction and mutation screening. The protocol of this study adhered to the provisions of the Declaration of Helsinki.

An altered phosphodiesterase activity within photoreceptors, which leads to an elevation in cyclic guanosine monophosphate (cGMP) levels, has been suggested as a possible disease mechanism.[1,3,7] However, the only change reported so far is a G→C nucleotide transversion in the 5′ UTR of the cone cGMP-phosphodiesterase γ subunit (*PDE6H*) gene, which caused a significant increase in reporter-gene transcription.[8] We screened the 5′ UTR and coding region of *PDE6H* in a panel of six unrelated patients of varied ethnic origin, using the primer pairs listed in table 1, but failed to detect any changes. Two flanking STR markers (*D12S320* and *D12S1669*) and two intragenic SNPs (*rs11056264* and *rs3748304*) flanking *PDE6H* also failed to cosegregate with disease in a family containing multiple affected members (data not shown). We conclude, therefore, that *PDE6H* is not the disease gene in our study sample.

To extend the search for the disease gene, we performed homozygosity mapping on a consanguineous family with six affected members, using the GeneChip Human Mapping 10K SNP Array (Affymetrix). The family was of Middle Eastern ethnicity, and the affected individuals were from three sibships of two generations, each sibship having parents who were first or second cousins (family 1 in figs. 2 and 3). A region containing the largest number of contiguous identical homozygous genotypes for all five affected individuals consisted of 17 SNPs within 0.98 cM (1.5 Mb) on chromosome 9p24, flanked by *rs1112534* and *rs1074449*. Identical homozygous genotypes occurred for 2,579 of 9,769 autosomal SNPs, and contiguity of 17 would not be expected to occur by chance in <5,000 experiments; all other contiguous regions contained ≤10

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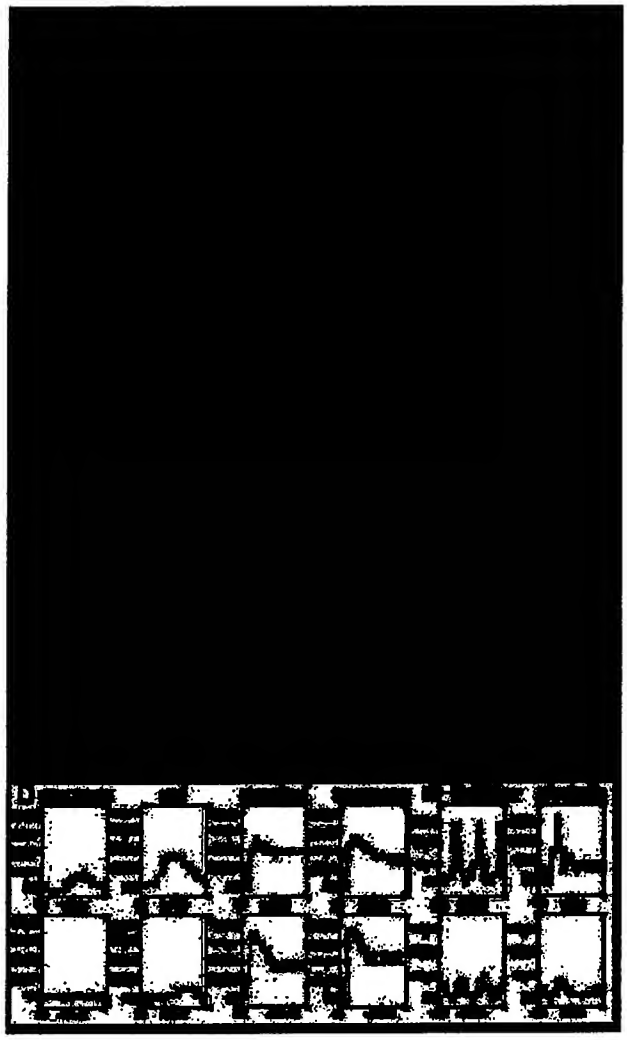


**Figure 1.** Autofluorescence (*A*) and color image (*B*) of right and left retina of affected patient aged 59 years (family 4), showing areas of central atrophy of retinal pigment epithelium (RPE) and choroid. *C*, Color image of right and left retina of affected patient aged 24 years (family 1), showing subtle RPE depigmentation around fovea and crystals at the macula. *D*, Full-field ERGs (International Society for Clinical Electrophysiology of Vision [ISCEV]) in an unaffected subject (*top row*) and in a representative patient from family 9 (*bottom row*). In the patient, dark-adapted responses to the dimmest flash (0.002 candela [cd]-s/m$^2$) are undetectable. Increasing stimulus intensity ("rod" 0.012 cd-s/m$^2$) produces an abrupt increase in amplitude and a delayed rod ERG. At higher flash energies ("standard" 3.0 cd-s/m$^2$ and "maximum" 11.5 cd-s/m$^2$), the a-wave commences normally but develops a broadened trough before a high-amplitude, sharply rising b-wave that approaches the upper limit of normality (supernormal). Flicker and photopic single-flash ERGs were performed after 10 min of light adaptation. ISCEV-standard 30-Hz flicker ERGs show delay and marked reduction. The photopic single-flash ERG is delayed and subnormal, with a simplified waveform and delayed recovery after the beta-wave. Broken lines replace blink artifacts, frequently seen after the ERGs with strong flashes.

SNPs. This finding supported the hypothesis of autozygosity in affected individuals for a disease locus at 9p24. The region contained seven known human expressed sequences (Ensembl version 37), including an attractive candidate—a voltage-gated $K^+$ channel subunit gene, family V, member 2 (*KCNV2* [MIM 607604]; synonyms are *Kv8.2*, *Kv11.1*, and *MGC120515*). A BLAST analysis of *KCNV2* mRNA against human dbEST identified 18 matching ESTs derived from ocular tissue, a higher proportion than in any other organ.

The two exons and intron-exon boundaries of *KCNV2* were amplified using the primer pairs listed in table 1. Direct sequencing of the amplified fragments was performed on an ABI 3100 with the BigDye Terminator v3.1 Cycle Sequencing Kit (PE Applied Biosystems). Genomic DNA from probands from 11 unrelated families who showed typical clinical features of the disorder (a detailed clinical description of a subset of these patients has been presented elsewhere[5]) was sequenced to determine variants. The ethnicities of the families were as follows: white, from the United Kingdom (families 4, 7, 8, 10, and 11); Middle Eastern (families 1 and 9); and South Asian (families 2, 3, 5, and 6). Numerous sequence variants were found, including five distinct nonsense variants, one in-frame deletion of 9 nt, and six distinct missense variants (fig. 3). Family 8 was heterozygous for three variants—a nonsense (Gln145Stop) and two missense (Asp147Phe and Ala259Val) changes—and cloning of a fragment amplified from exon 1 into pGEM-T-Easy vector, followed by sequencing with the use of vector-specific primers, showed Asp147Phe to be in *cis* with the nonsense mutation. One proband (family 10) showed only a single heterozygous change. Finally, one proband (family 11) showed no sequence variants, and it remains to be seen whether this family has undetected mutations or represents genetic heterogeneity for this disorder. Appropriate segregation was demonstrated in three families (1, 2, and 7) in which multiple members were affected and were available for analysis. Detailed electrophysiology was performed with the two heterozygous parents of family 6, and no subclinical abnormalities were detected, suggesting that heterozygosity for mutation in *KCNV2* does not produce a clinical effect.

*KCNV2* (*Kv11.1*) was identified elsewhere, through a homology search of the human genome for voltage-gated $K^+$ channel subunit genes.[9] The KCNV2 protein comprises 545 aa and, in common with other voltage-gated $K^+$ channel subunits,[9,10] comprises an N-terminal A and B box (NAB) or T1 domain and six transmembrane (TM) domains (S1–S6), with a $K^+$ selective motif, GlyTyrGly, in the pore-forming loop (P loop) between S5 and S6. A highly charged S4 segment, which bears a regular array of positively charged amino acids (Arg or Lys) in every fourth position, is the principal structural element responsible for voltage sensing. A BLASTN search of genomes with the human KCNV2 protein sequence identified orthologues in seven other mammals (two primates, dog, cow, mouse,

**Table 1. PCR Primer Pairs for *PDE6H, KCNV2, KCNB1, KCNC1,* and *KCNF1***

| Gene and Primer Pair | Exon | Size (bp) | Primer (5′→3′) Forward | Reverse |
|---|---|---|---|---|
| *PDE6H*: | | | | |
| A | 1 | 290 | GGTCCCCATGACTTGAAAGATC | AAACAAAAGCAGGTTTTGTGGG |
| B | 2 | 159 | TCTCCATGTGAGTGACTCCAA | GCAGAACTCCAAGTGCGAAGT |
| C | 3 | 328 | AGGAGTGAAGTGTCTCTGCCT | CAGTAAGAGAACTCTTAGTAG |
| *KCNV2*: | | | | |
| A | 1 | 1,133 | TAGAGGCAGTGAGCAGGTGA | CACTCGAGCAGCAGCTGA |
| B | 1 | 826 | TAGAGGCAGTGAGCAGGTGA | TCTCCATGAGGTTCCAGAGG |
| C | 1 | 1,184 | GCTGGACTACTGCGAGCTG | ATCCATCGCCGTTTTGTTAG |
| D | 1 | 822 | AGGAACTCTTCCGCGACAT | ATCCATCGCCGTTTTGTTAG |
| E | 2 | 448 | GCTTGCTCCTCTCCCTTTCT | CATTTCCTTTTGCTGCCAAT |
| F | 1 | 731 | GCTGGACTACTGCGAGCTG | CACTCGAGCAGCAGCTGA |
| G* | 1 | 427 | GCTGGACTACTGCGAGCTG | TCTCCATGAGGTTCCAGAGG |
| *KCNB1*: | | | | |
| H | ... | 424 | GGGAGCGGGAAGGCGAGGAGTT | CTGGACCACGCGGCGGACATTC |
| *KCNC1*: | | | | |
| I | ... | 566 | TGGCCTTCGCTTCCCTCTTCTTCA | GCTCACTGGCGCTGGGGTCATTG |
| *KCNF1*: | | | | |
| J | ... | 536 | TGCTGGCCATCCTCCCCTTCTACG | CGCCCCCGCTGCTGGAGTTGAG |

* Primer pair G was used for the amplification of a *KCNV2* gene fragment, for the generation of riboprobes for in situ hybridization.

rat, and opossum), in chicken, in frog, and in three fish species. The two-exon structure and the position of the single intron was preserved in all. A ClustalW analysis of all 13 sequences demonstrated a high degree of amino acid identity with the human sequence, ranging from 95% with the other primates to 60% with fish.

All five nonsense mutations occur in exon 1, and mRNA would be predicted to succumb to nonsense-mediated decay. If translated, the mutated sequences all would lead to termination before four of the TM domains and the P loop (fig. 3). The Gly459Asp variant, found on both alleles of a proband of Pakistani origin (family 6), disrupts the

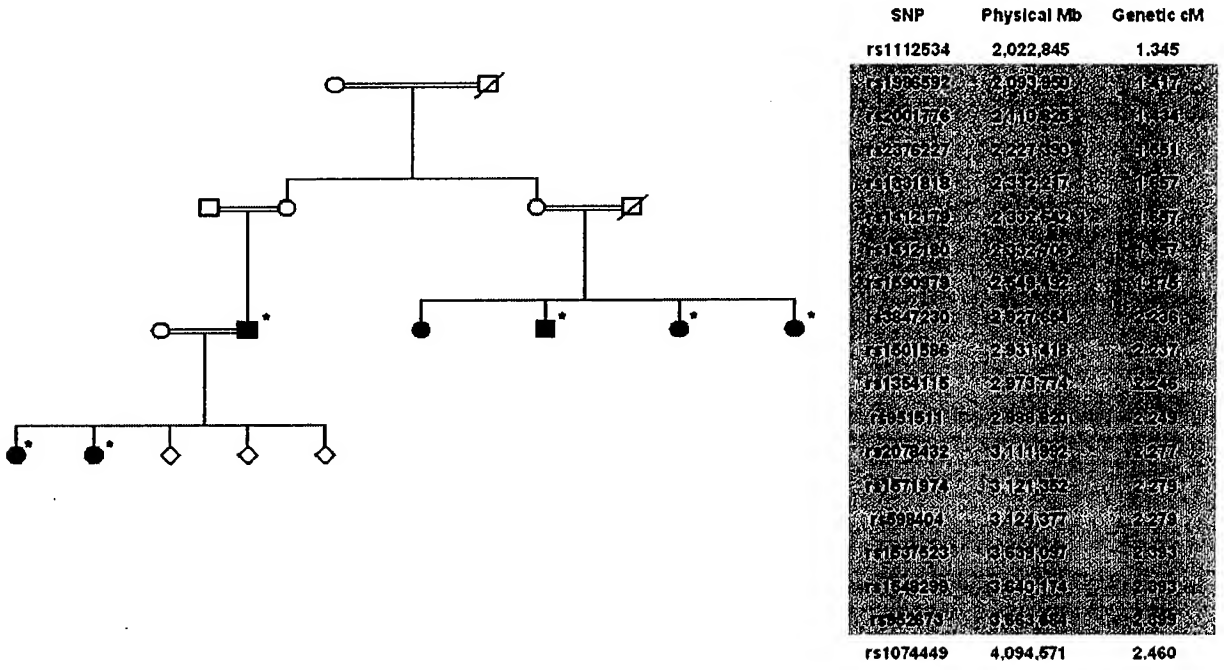
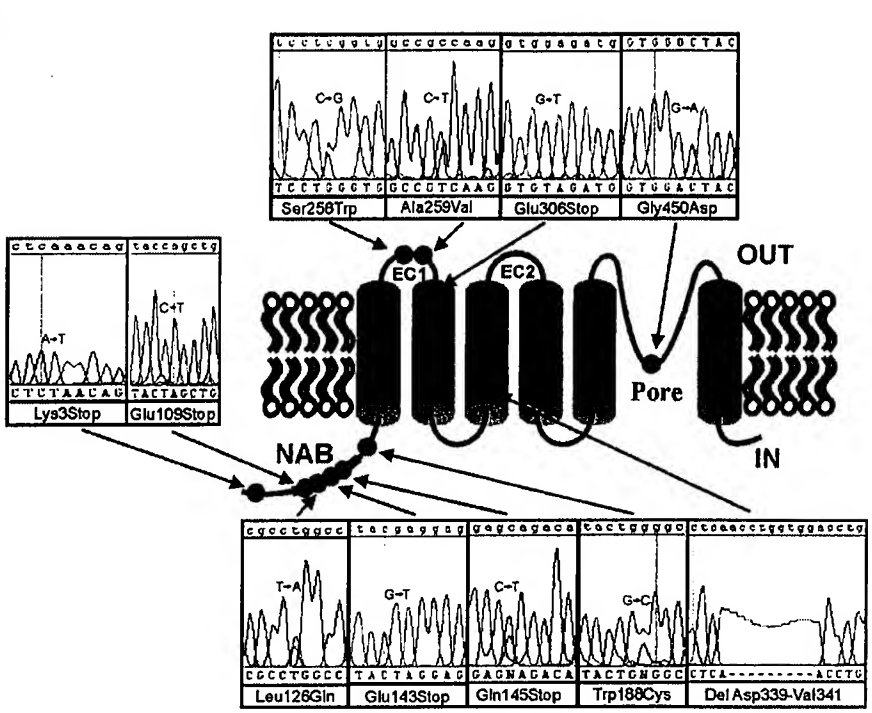



**Figure 2.** Linkage analysis. *A,* Pedigree of consanguineous family used for mapping with the GeneChip Human Mapping 10K SNP Array (Affymetrix). Individuals included in the analysis are marked by an asterisk. *B,* Contiguous homozygous SNPs at chromosome 9q24 (*shaded*), with flanking SNPs above and below.

A




B

| Family | Number of affected individuals | Nucleotide changes | Amino acid changes | Protein domains |
|---|---|---|---|---|
| 1 | >6 | G427T | Glu143Stop | NAB |
| 2 | 2 | G916T | Glu306Stop | S2 |
| 3 | 1 | C325T | Gln109Stop | NAB |
| 4 | 1 | A7T | Lys3Stop | N-terminus |
| 5 | 1 | Del 1015-1024 ACCTGGTGG | Del Asp339-Val341 | S3 |
| 6 | 1 | G1376 A | Gly459Asp | Pore |
| 7 | 2 | G427T/C767G | Glu143Stop/Ser256Trp | NAB/EC1 |
| 8 | 1 | C430T/C776T | Gln145Stop/Ala259Val | NAB/EC1 |
| 9 | 1 | G427T/G564C | Glu143Stop/Trp188Cys | NAB/N-terminus |
| 10 | 1 | T377A/? | Leu126Gln/? | NAB |

**Figure 3.** Mutations in KCNV2 channel protein. *A*, Domain structure of KCNV2, with the approximate position of mutations (*circles*) linked to electropherograms showing sequence in patient DNA. *B*, KCNV2 disease-associated mutations. Affected individuals from families 1–6 were homozygotes for the variant indicated. Only a single missense mutation was found in family 10; the "missing" mutation is indicated by a quotation mark. Additionally, the proband of family 4 was heterozygous for L533V, and the proband of family 8 also had D147F in phase with Q145X.

first amino acid of the highly conserved GlyTyrGly $K^+$ selective motif in the P loop. The four missense variants—Leu126Gln, Trp188Cys, Ser256Trp, and Ala259Val—and the first two residues of the AspLeuVal deletion occur at sites that are conserved across all 13 vertebrate orthologues and that are each highly conserved in other voltage-gated $K^+$ subunits.[10] Two other missense variants, Asp147Phe and Leu533Val, are not conserved to the same degree.

The portion of exon 1 harboring the variants between codons 126 and 188 was sequenced in 72 control samples (144 control chromosomes) derived from unexamined, anonymous U.K. blood donors (European Collection of Cell Cultures [ECACC] human control panel Q5671). All 144 alleles had Leu at codon 126 and Trp at codon 188. Codon 147 was polymorphic; two alleles showed an Asp→Gly (GAC→GGC) change, and one allele showed an Asp→Glu (GAC→GAG) change. No other variants were seen. Specific assays were designed to test for Ser256Trp and Ala259Val (KBiosciences Amplifluor) and were run on 2 × 96 ECACC unexamined human control panels. The wild-type variant was present in all 384 alleles. Finally, the

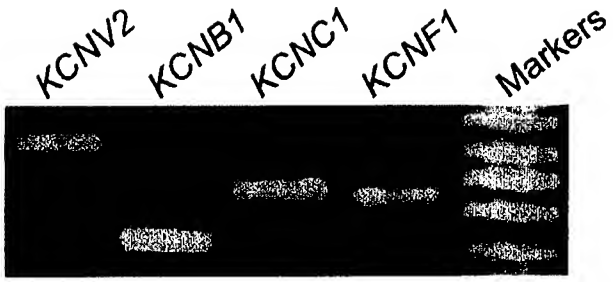



**Figure 4.** Gel image showing PCR products of *KCNV2*, *KCNB1*, *KCNC1*, and *KCNF1* amplified from human retinal cDNA. The fragments were amplified using primer pairs G, H, I, and J (table 1), were cloned into the pGEM-T-Easy vector, and were sequenced to confirm identity.

Gly459Asp variant was tested with 50 unexamined control individuals of Pakistani ethnicity, and all 100 chromosomes showed the wild-type allele. Given the sample sizes used in the examination of controls, this would give an 80% probability of detecting one or more alleles with minor frequency 0.016 (100 chromosomes), 0.011 (144 chromosomes), and 0.004 (384 chromosomes), calculated using the binomial distribution. The Leu533Val variant, found as a heterozygous change in the proband of family 4, is a known coding-region SNP (*rs12352254*). The Asp147Phe variant is also likely to be a rare polymorphism, since this codon was found to be polymorphic in 3 of 144 alleles, and is likely to be in *cis* with a second rare variant Gln145Stop (see above).

Presence of the *KCVN2* transcript has been demonstrated, with the use of RT-PCR, in a variety of human tissues,[10] although, to our knowledge, retinal tissue has not been tested previously. We amplified the coding sequence from human retinal cDNA and confirmed the expression of *KCNV2* (fig. 4). Within the human retina, in situ hybridization with a *KCNV2* antisense riboprobe showed a positive signal in the inner segments of both cone and rod photoreceptors, which was not seen with the control sense probe (fig. 5).

Although mutations in genes encoding voltage-gated $K^+$ channel subunits have been shown elsewhere to underlie other human diseases,[11–13] this is the first report of a disorder affecting the visual system. Such channels are formed as a tetramer, and KCNV2 belongs to the group of electrically silent channels that do not produce a $K^+$ current as a homotetramer but interact with other $K^+$ channel subunits—such as KCNB1, KCNC1, and KCNF1—to form functional heterotetramers.[10] It is, therefore, likely that KCNV2 binds with at least one other subunit to form channels in photoreceptors. Gene-specific primers (table 1) successfully amplified *KCNB1*, *KCNC1*, and *KCNF1* fragments from human retinal cDNA (fig. 4), and KCNB1 subunits have been localized previously to inner segments of rods.[14] All three subunits are, therefore, candidates for interaction with KCNV2, although other subunits remain to be tested.

A $K^+$ current, $I_{Kx}$, which is present in vertebrate rod photoreceptors and deactivates slowly when the cell is hyperpolarized, figures prominently in setting the dark resting potential and accelerating the voltage response to small photocurrents.[15,16] The process that underlies this current may be perturbed or abrogated by the loss of KCNV2 subunits. The presence of cone dysfunction in our patients would imply that both cones and rods require functional voltage-gated $K^+$ channels, and further studies should improve our understanding of the exact role of *KCNV2* in phototransduction and in disease pathogenesis.

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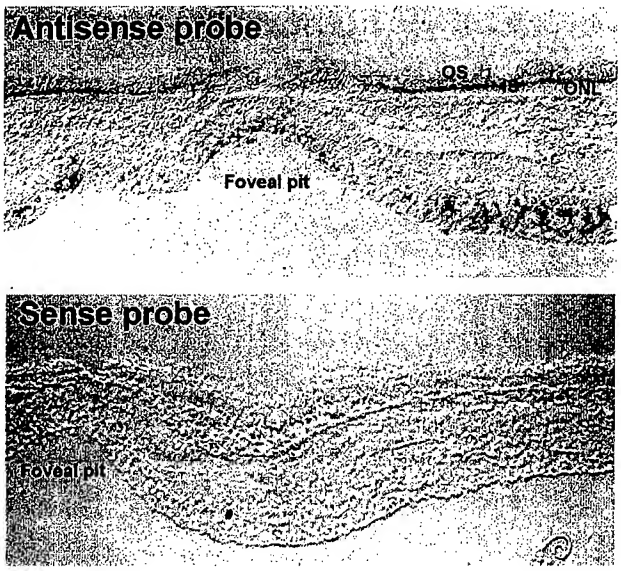


**Figure 5.** In situ hybridization of human retina probed with antisense and sense *KCNV2* riboprobes. Sections 10 $\mu$m thick, from fixed and cryoprotected human retinal tissue, were probed with either antisense or sense digoxigenin (DIG)–labeled riboprobes generated from a 427-bp gene fragment, cloned into pGemT Easy vector, that spans the central portion of exon 1. Retinal sections were prepared for hybridization with the use of standard methods. Hybridization and washing was performed at 65°C. Signal was resolved using anti-DIG antibody at a 1:2,000 dilution, followed by color development. OS = Photoreceptor outer segment; IS = photoreceptor inner segment; ONL = outer nuclear layer.

valuable English-Arabic translation; and Bev Scott (UCL Institute of Ophthalmology), for technical assistance. We also thank London IDEAS, for the use of the genotyping facility for the full-genome screen of the large consanguineous family, and Professor Pete Scambler and Kerra Pearce (UCL Institute of Child Health), for their technical advice and expertise.

## Web Resources

The URLs for data presented herein are as follows:

BLAST, http://www.ncbi.nlm.nih.gov/BLAST/
ClustalW, ftp://ftp.ebi.ac.uk/pub/software/unix/clustalw/
ECACC, http://www.ecacc.org.uk/ (for control DNAs from anonymous white donors)
Ensembl, http://www.ensembl.org/
International Society for Clinical Electrophysiology of Vision (ISCEV), http://www.iscev.org/
London IDEAS, http://www.londonideas.org/internet/professionals/genotyping_service/index.html

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# Exhibit C

# A novel potassium channel gene, *KCNQ2*, is mutated in an inherited epilepsy of newborns

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**Idiopathic generalized epilepsies account for about 40% of epilepsy up to age 40 and commonly have a genetic basis. One type is benign familial neonatal convulsions (BFNC), a dominantly inherited disorder of newborns. We have identified a sub-microscopic deletion of chromosome 20q13.3 that co-segregates with seizures in a BFNC family. Characterization of cDNAs spanning the deleted region identified one encoding a novel voltage-gated potassium channel, KCNQ2, which belongs to a new KQT-like class of potassium channels. Five other BFNC probands were shown to have *KCNQ2* mutations, including two transmembrane missense mutations, two frameshifts and one splice-site mutation. This finding in BFNC provides additional evidence that defects in potassium channels are involved in the mammalian epilepsy phenotype.**

Benign familial neonatal convulsions (BFNC; OMIM 121200) are an autosomal-dominantly inherited epilepsy of the newborn. This idiopathic, generalized epilepsy typically has an onset of seizures on days 2–4 of life. Spontaneous remission of the seizures occurs between two and fifteen weeks[1–3]. Seizures typically start with a tonic posture, ocular symptoms and other autonomic features, which then often progress to clonic movements and motor automatisms. These neonates thrive normally between the seizures, and their neurological examinations and later development indicate normal brain function[1–3]. Despite normal neurological development, however, seizures recur later in life in approximately 16% of BFNC cases, compared with a 2% cumulative lifetime risk of epilepsy in the general population[1–3].

Linkage analysis in a large kindred demonstrated that a gene responsible for BFNC maps to chromosome 20q13.3, close to the markers *D20S20* and *D20S19* (ref. 4). Soon after the initial report, two centres confirmed linkage of BFNC to the same two genetic markers on chromosome 20, termed the *EBN1* (epilepsy benign neonatal type 1) locus[5–7]. A more distal marker, *D20S24*, shows complete co-segregation with the BFNC phenotype in chromosome-20–linked families. Finding a distal flanking marker for the BFNC locus has not been successful, probably because of its proximity to the telomere. This telomeric region is characterized by a high recombination rate between markers when compared with the physical distance[7]. In fact, Steinlein *et al.* have demonstrated that the three markers *D20S19*, *D20S20* and *D20S24* are contained on the same 450-Mb *MluI* restriction fragment[7].

A second chromosomal locus, *EBN2*, has also been identified for BFNC. Lewis *et al.*[8] demonstrated linkage to markers on chromosome 8q24 in a single Mexican-American family affected with BFNC. Evidence for this second locus was also reported in a Northern European pedigree[9]. All of the families in the present study show linkage to chromosome-20q markers with lod scores of greater than 3.0 or have probands with clinical manifestations consistent with BFNC[10]. To find the gene responsible for BFNC, we narrowed the BFNC region with a sub-microscopic deletion in a single family, identified candidate cDNAs in this deletion and then searched for mutations in other BFNC families.

## Deletion of a critical region in a BFNC family

Evidence for a small deletion first came from a genotypic observation with a three-allele RFLP marker, *D20S24*. Analysis of one family, kindred 1547, revealed that a null allele occurred exclusively in individuals with BFNC and in two individuals previously shown to be non-penetrant with the VNTR markers *D20S20* and *D20S19* (Fig. 1). The existence of a deletion co-segregating with the BFNC phenotype in this family was confirmed by fluorescence *in situ* hybridization (FISH) in cell lines of kindred 1547 individuals using as probes the *D20S24* plasmid and two genomic P1 clones containing this marker. The FISH analysis was performed on a single affected individual from K1547 (III-10), a kindred in which affected family members have seizures starting on day 3 or 4. The individual III-10 presented with seizures beginning at three days, and 118 generalized seizures were observed until the age of 23 days. A single seizure was observed at three and a half months in conjunction with an acute infection of the middle ear and fever. No seizures were observed in this individual thereafter. The FISH analysis was car-

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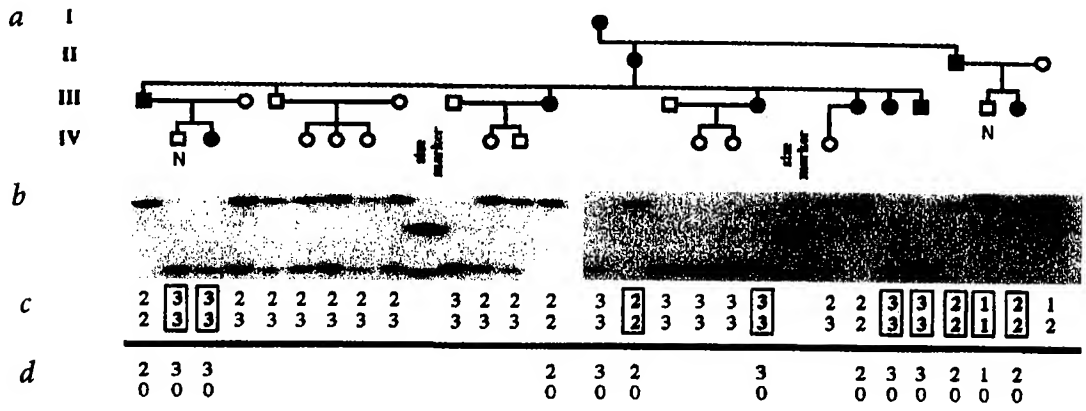


**Fig. 1** A deletion was inferred from a null *D20S24* allele in affected individuals. **a**, Kindred 1547: filled symbols represent affected individuals; N, non-penetrant individuals. **b**, Southern blot of kindred 1547 genomic DNA digested with *Taq*I and probed with *D20S24*; the size marker bands are 4.3 kb and 3.2 kb. **c**, Genotypes with misinheritances shown in boxes. **d**, Corrected genotypes.

ried out blinded with respect to affection status. The analyst examined 36 metaphase spreads, and in the 28 in which a signal was detected, only one chromosome-20 homologue showed hybridization to the *D20S24* plasmid. The 12-kb probe thus gave a hybridization efficiency of 78%. 93% of chromosome spreads of normal individuals exhibited labelling of both chromosomes using the 12 kb *D20S24* probe. The deleted chromosome-20 homologue was identified by G-banding of the chromosomes. These FISH data confirm the existence of a putative null allele shown (Fig. 1) to co-segregate with all affected individuals in the pedigree. Although the 12-kb *D20S24* probe was deleted on one chromosome in affected individuals, the overlapping P1 clones (80 kb in size), which together span approximately 130 kb, showed a positive FISH signal, indicating that the deletion is smaller than 130 kb (Fig. 2).

## Identification of cDNAs in the deleted region

Using the same probes, we identified cDNAs in the region of the deletion by screening a fetal brain cDNA library. A single cDNA isolated with the *D20S24* probe showed significant homology with *KVQT1* (*KCNQ1*), the chromosome-11 potassium-channel gene responsible for the long-QT syndrome and the Jervell and Lange-Nielsen cardio-auditory syndrome[11–13]. Homology extended from amino acid 511 to 562 of *KCNQ1*. A second probe of the fetal brain cDNA library with P1-KO9-6b resulted in the isolation of two additional cDNAs, which showed significant homology with *KCNQ1* amino acids 398–406 and 354–378, respectively. Additional sequence encoding this gene, named *KCNQ2*, was obtained from RACE experiments with adaptor-ligated double-stranded cDNA from fetal and adult brain tissue and from other cDNA clones isolated from a temporal cortex cDNA library.

Composite clones encoding 872 amino acids of *KCNQ2* have been isolated (Fig. 3). The putative initiator methionine lies within a region similar to the Kozak consensus sequence[14]. *KCNQ2* encodes a highly conserved six-trans-membrane motif as well as a pore region, hallmarks of a $K^+$ ion channel gene. The S2, S3 and S4 trans-membrane regions also contain charged amino acids that are found in all members of the $K^+$ channel sub-families, including *Shaker*, *Shab*, *Shaw* and *Shal*. A search of GenBank with *KCNQ2* sequence shows identical nucleotide sequence to *HNSPC*, a 393-amino-acid putative potassium-channel cDNA isolated from a human neuroblastoma cell line[15]. However, the last 21 amino acids of *HNSPC*, including a stop codon, are encoded by a sequence that in *KCNQ2* is intronic. A search of the human expressed-sequence-tag database (dBest) shows seven clones encoding portions of *KCNQ2*. Wei *et al.* have identified a gene from *Caenorhabditis elegans*, *nKQT1*, that appears to be a homo-

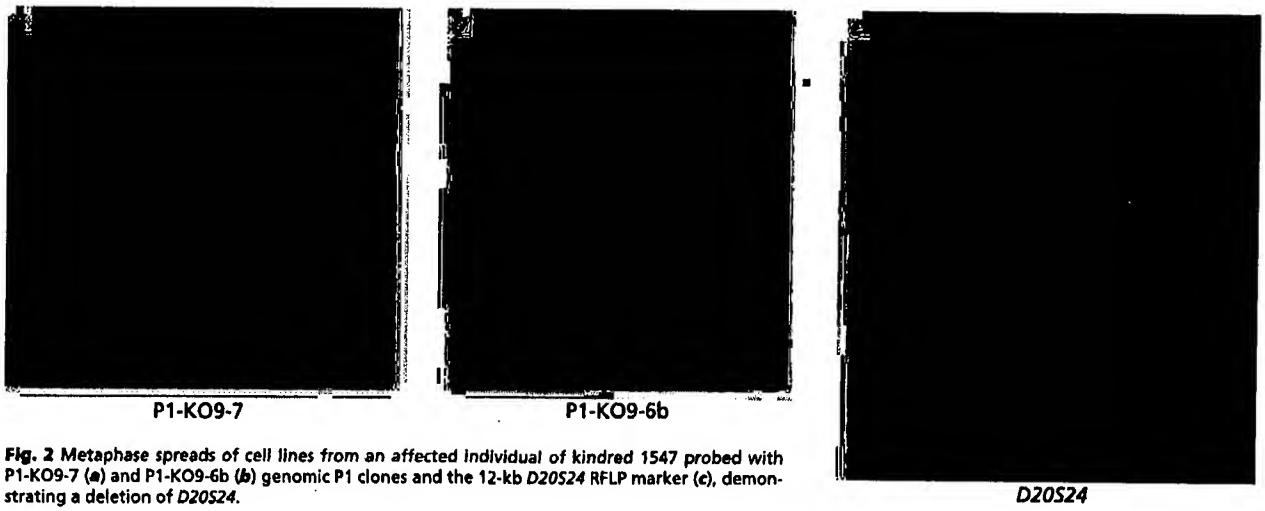


**Fig. 2** Metaphase spreads of cell lines from an affected individual of kindred 1547 probed with P1-KO9-7 (**a**) and P1-KO9-6b (**b**) genomic P1 clones and the 12-kb *D20S24* RFLP marker (**c**), demonstrating a deletion of *D20S24*.

**Table 1 • Mutations in *KCNQ2* in BFNC families**

| Mutation | Region | Kindred | Controls | Nucleotide change |
|---|---|---|---|---|
| deletion | n/a | K1547 | 70 | n/a |
| 283insGT | pore | K1504 | 70 | frameshift |
| Y284C | pore | K3904 | 70 | TAC to TGC |
| A306T | S6 | K1705 | 70 | GCG to ACG |
| 522del 13 | C-terminal | K3369 | 70 | frameshift |
| 544-1G to A | C-terminal | K3933 | 70 | splice-site variant |

n/a, not available.

logue of *KCNQ2* (ref. 16). This group also described the human EST homologue of *nKQT1*, *hKQT2*, which is a partial clone of *KCNQ2* (ref. 16). In addition to the six trans-membrane domains and the pore, a small region 5′ of trans-membrane domain S1 is also conserved between *KCNQ2*, *KCNQ1* and *nKQT1*. Unlike other $K^+$ channel sub-families, the C-terminal domain appears to contain highly conserved residues for *KCNQ2*, *nKQT1* and *KCNQ1* (Fig. 3). 3′–RACE has not led to the identification of the poly-A tail or the polyadenylation signal, although 486 nucleotides have been obtained after the termination codon.

### *KCNQ2* expression and mutations in BFNC patients

The *KCNQ2* cDNA hybridizes to transcripts approximately 1.5, 3.8 and 9.5 kb in size on northern blots made from brain (data not shown). The 1.5- and 9.5-kb transcripts appear to be expressed in both adult and fetal brain. The 3.8-kb transcript is expressed in select areas from adult brain, particularly in the temporal lobe and the putamen.

Mutational analysis of *KCNQ2* was performed on one affected individual from each of our twelve BFNC families or unrelated probands. In addition to the substantial deletion in kindred 1547, mutations were identified in five other BFNC families. Mutational analysis was carried out by first screening probands for SSCP variants and then sequencing each proband's DNA to determine the basis for the molecular variation. Mutations identified include two missense mutations, two frameshift mutations and one splice-site mutation (Table 1). None of the mutations identified were seen in SSCP analysis of our panel of 70 unrelated, unaffected individuals. In our collection of twelve BFNC probands, three (K1504, K1547, K1705) were from families that showed significant linkage, with lod scores greater than 3.0 on chromosome 20 (ref. 10). Mutations were found in all three families. Two were single probands, and a splice-site mutation was found in one (K3933). The remaining seven families were too small for linkage to chromosome 20 to be demonstrated. In this group, mutations in *KCNQ2* were observed in two families (K3904 and K3369). The complete *KCNQ2* gene has not been screened for mutations in the six remaining pedigrees. Furthermore, mutations were shown to segregate completely with affection status in all of the BFNC families in which mutations were identified. An example of this segregation is shown in Fig. 4 for the 2-bp insertion identified in kindred 1504; all eleven affected members have the SSCP variant, and all seven unaffected individuals have wild-type SSCP bands. Two neutral polymorphisms were identified in our 70 control individuals (CEPH parents)—one in codon 304 (TTC→TTT) in the S6 trans-membrane domain, with a frequency of 7.0%, and a second in codon 573 (GCC→GCT) in the 3′ region of *KCNQ2*, with a frequency of 0.58%.

It is predicted that the splice-site mutation in the conserved 3′ region of *KCNQ2* and the two frameshift mutations, one in the pore region and one before the highly conserved 3′ region, lead to altered protein products. In the case of the 283insGT pore mutation, a predicted stop codon is found 36 amino acids downstream; in the case of the 522del13 3′ mutation, a predicted stop codon is found two amino acids downstream. Also, the 2-bp insertion mutation, 283insGT, would lead to a completely altered S6 trans-

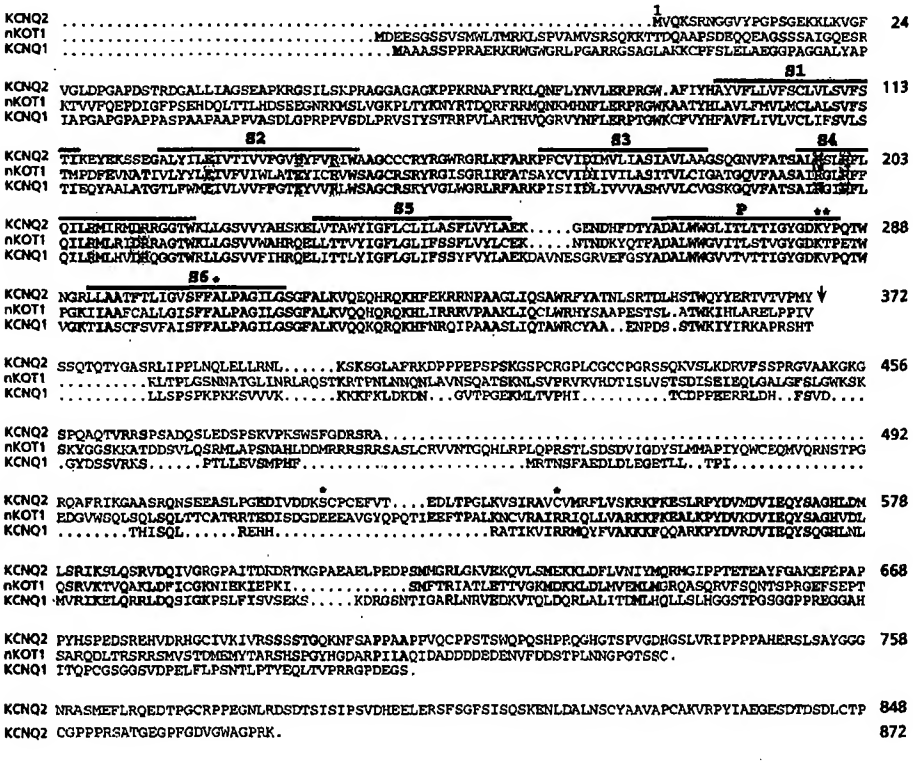


**Fig. 3** Amino-acid alignment of KCNQ2 with KCNQ1 and nKQT1 from *C. elegans*. The arrow indicates the exon–intron boundary that is read through in the *HNSPC* clone; an asterisk indicates the sites of mutations listed in Table 1. KCNQ2 and KCNQ1 share 60% identity and 70% similarity in the trans-membrane regions from S1 to S6. Grey shading indicates conserved charged amino acid residues in S2, S3 and S4.

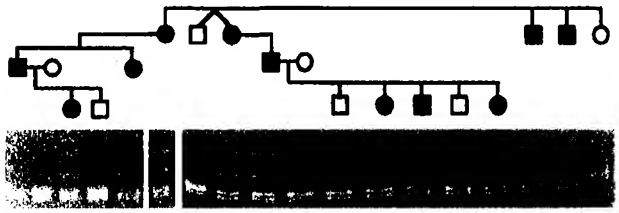

**Fig. 4** Segregation of SSCP variants in the KCNQ2 pore region in kindred 1504. Sequence analysis revealed the existence of a 2-bp insertion in affected individuals showing the upper two (variant) bands.

membrane domain. Although the breakpoints of the kindred-1547 deletion have not been determined, it is known that the 12-kb plasmid, which includes the RFLP marker locus, *D20S24*, contains at least 80 codons (residues 509–588 of *KCNQ2*) of sequence from the highly conserved 3′ region of the *KCNQ2* gene, indicating that at least this portion of the gene is deleted in affected kindred-1547 individuals. The deletion in this family may also include other cDNAs. The two missense mutations change amino-acid residues in key functional domains, the pore and S6 domains. Six unique mutations have been identified in *KCNQ2* to date. The mutation defined by a 13-bp deletion at amino-acid 522 in kindred 3369 is of interest in that there is a greater variation in the reported clinical ages at onset within this family than in typical BFNC families. In kindred 3369, three individuals had onset of seizures within the first two weeks of life, whereas three individuals had onset of seizures at three, four and five months of age.

## Discussion

This is the first human idiopathic generalized epilepsy in which a $K^+$ channel has been implicated. BFNC is considered to be a true idiopathic epilepsy because seizures are the only manifestation observed. This is in contrast to other epileptic syndromes, which have degenerative characteristics and an epileptic component, such as progressive myoclonus epilepsy of the Unverricht-Lundborg type. It is not surprising, therefore, that an alteration in a gene that directly regulates neuronal excitability could produce the epileptic disorder seen in BFNC. Voltage-gated potassium channels repolarize neuronal membranes that have been depolarized by $Na^+$ and $Ca^{++}$ voltage-gated ion channels. $K^+$ channels are also thought to repolarize neuronal membranes after activation of excitatory neurotransmitter ion channels, including glutamate and acetylcholine. In the presence of mutant KCNQ2 channels with reduced function, excitatory ligand and voltage-gated channels that are activated would remain open longer[17–19]. Such unchecked activity of excitatory systems could lead to an epileptic phenotype. Additional studies using brain slices and whole animal models have implicated altered $K^+$ regulation as having a causal role in epilepsy[19]. Electrophysiological analysis of the mutant KCNQ2 channels will shed light on how the mutations identified in the current study produce an epileptic phenotype. It is likely that KCNQ2 will have biophysical properties similar to the delayed rectifier KCNQ1 channel. KCNQ1 alpha subunits co-assemble with minK beta subunits to form heteromultimeric $I_{Ks}$ channels in the heart[20]. It is possible that KCNQ2 subunits co-assemble with minK-like beta subunits in the brain. This interaction may also alter the gating properties of the resulting heteromultimeric channel, as is the case for KCNQ1.

Mutations in $K^+$ channels have been associated with epilepsy in only one other case—the weaver mouse, in which a single missense mutation in the *GIRK2* gene produces spontaneous seizures[21,22]. Mutations in $K^+$ channels have been implicated in other human disorders, such as the long-QT syndrome on chromosome 11 and ataxia/myokymia on chromosome 12 (refs 11,13,23–25). Long-QT syndrome is associated with four loci, two of which are the $K^+$ channel genes *HERG* and *KCNQ1*. In *KCNQ1*, mutational hot spots have been identified in the pore and S6 domains, where missense mutations account for most of the disease-causing mutations in LQT[11,23].

The mutation in the BFNC kindred 1705 is an alanine→threonine substitution in the S6 trans-membrane segment. This alanine residue is conserved in all members of the *Shaker, Shab, Shaw* and *Shal* sub-families of potassium channels identified to date[26,27]. The *KCNQ1* gene, to which the *KCNQ2* ion channel is most closely related, also contains an alanine in this position. In six unrelated LQT1 families, the disease-causing mutation occurs at the same position, where the alanine is changed to a valine[11,23]. This S6 trans-membrane domain has been shown to be involved in $K^+$ ion permeation in the *Shaker* subtype[28], and it may serve a similar function in *KCNQ2*. The C-terminal region appears to be important for gene function because a 13-bp deletion and a splice-site mutation both produce an epileptic phenotype in separate BFNC families (Table 1, Fig. 3). The same region is known to have a deletion–insertion mutation in *KCNQ1* in individuals with the Jervell and Lange-Nielsen recessive form of LQT and associated deafness[13]. Disease-causing mutations in the C-terminal region further argue for a functional protein encoded by the *KCNQ2* gene rather than the shorter *HNSPC* clone.

The pore region of $K^+$ ion channels belonging to the same structural class have been characterized extensively by mutational analysis[29]. The 2-bp insertion observed in kindred 1504 occurs immediately after the universally conserved GYG motif. An insertion here not only alters the length of the pore that is believed to be crucial for function[29–32] but also modifies the signature sequence of the pore and produces a truncated protein.

In infants of families that have been linked to the chromosome-20 form of BFNC, EEG recordings show initial suppression of activity throughout the brain, followed by generalized discharges of spikes and slow waves[1–3]. In adults, the *KCNQ2* gene is expressed in various parts of the brain. Cortical regions as well as sub-cortical areas, such as the thalamus and caudate nucleus, contain transcripts of *KCNQ2* of various sizes (data not shown). It is possible that this expression pattern is also the same in the newborn infant.

The close homology (60% identity and 70% similarity of amino acids in the trans-membrane region from S1 to S6) of *KCNQ2* with *KCNQ1* and with the *C. elegans nKQT1* gene and the reduced homology of these channels with the *Shaker, Shab, Shaw* and *Shal* sub-families imply that they belong to a unique family of $K^+$ ion channels, called KQT-like by Wei *et al.*[16]. A new $K^+$ ion channel now known to be expressed in the brain raises the question of whether additional, undiscovered members of this gene family might be responsible for other forms of idiopathic, generalized epilepsies with tonic-clonic convulsions. A similar idiopathic seizure disorder seen early in development is benign familial infantile convulsions (BFIC). In BFIC, the seizures begin at four to eight months of age and remit after several years. BFIC maps to chromosome 19q in five Italian families[33,34]. It is reasonable to hypothesize that BFIC and the chromosome-8 form of BFNC are also caused by mutations in as yet unidentified members of the KQT-like family of $K^+$ ion channels or by minK-like proteins.

In conclusion, we have shown that a genetic defect of a potassium channel is associated with the human idiopathic epilepsy of BFNC. This finding adds to the growing list of channelopathies in humans[17,35,36] and suggests that drugs that directly or indirectly modulate $K^+$ ion channels will be helpful in the treatment of seizure disorders.

## Methods

**Southern-blot analysis.** Genomic DNA (5 μg) was cut with *TaqI* and transferred to a nylon membrane. Filters were hybridized overnight at 65 °C in PEG hyb (7% PEG, 10% SDS, 50 mM $NaPO_4$ and 200 μg/ml total human DNA) with the *D20S24* plasmid probe labelled by random priming (Stratagene). Filters were washed at 2× SSC, 0.1% SDS twice at room temperature, followed by one wash in 0.5 × SSC, 0.1% SDS at 65 °C.

**Fluorescence *in situ* hybridization.** Chromosomes from transformed lymphocytes were prepared with a 30-min ethidium-bromide treatment, followed by 3 h in Colcemid (CIBA). Cells were then pelletted and re-suspended in hypotonic solution (0.75 M KCl) for 20 min, followed by the addition of four or five drops of fresh fixative (3:1 methanol:acetic acid). Cells were again pelletted, vortexed and then carefully re-suspended in fixative. After three washes in fixative, metaphases were stored at 4 °C. Probe (400 ng) was labelled with biotin and hybridized to slides of metaphase spreads by means of standard hybridization procedures. Probes were then fluorescently tagged with avidin-FITC (Vector); the signal was intensified with biotin-labelled anti-avidin, followed by avidin-FITC. The chromosomes were then counterstained with DAPI and visualized with a Zeiss Axioplan fluorescent microscope equipped with FITC, DAPI and triple-band-pass filter sets. Images were captured with Probevision software (Applied Imaging) and photographs printed on a Kodak XL 7700 colour image printer.

**Full-length cDNA.** A total of $10^6$ clones of a fetal brain library (Stratagene) were probed sequentially with inserts from P1-KO9-6b and P1-KO9-7, and the plasmid *D20S24* was labelled by random priming (Stratagene) with $^{32}$P-dCTP (Du Pont–NEN). Hybridizations were performed in 5× SSC, 10× Denhardt's, 0.1 M $NaPO_4$ (pH 6.7), 100 μg/ml salmon sperm DNA, 0.1% SDS and 50% formamide. Blots were washed in 2× SSC, 0.1% SDS twice at room temperature, followed by one wash in 0.5× SSC, 0.1% SDS at 42 °C. To identify the full-length gene, 5′– and 3′–RACE were performed on adaptor-ligated fetal and adult brain cDNA (Clontech); PCR products were sub-cloned (Invitrogen) and sequenced on an ABI 377 (Advanced Biotechnologies). Additional sequence came from screening a temporal cortex cDNA library (Stratagene).

**Mutational analysis.** Coding regions S1–S6 and conserved regions in the 3′ end of *KCNQ2* were amplified by PCR with primers within introns and analysed by SSCP with 20% polyacrylamide gels containing TBE buffer (Novex) run at 4 °C. The exon–intron boundaries were identified by sequencing products obtained by exon–exon PCR on genomic P1 clones or directly from RACE products that contained unprocessed transcripts. PCR products showing variants seen on SSCP were either cloned and sequenced or re-amplified with M13 reverse and M13 universal-tailed primers and sequenced directly on an ABI 373 or 377 (Advanced Biotechnologies) with dye-primer chemistry.

**GenBank accession numbers.** The number for *KCNQ2* is AF033348; that for *HNSPC* is D82346.


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# Exhibit D

Expert Opinion

Monthly Focus: Central & Peripheral Nervous Systems

# KCNQ potassium channels: drug targets for the treatment of epilepsy and pain

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**Epilepsy and neuropathic pain are disorders characterised by excessive neuronal activity. These disorders are currently managed by drugs that are capable of dampening neuronal excitability, including voltage-gated sodium channel blockers, voltage-operated calcium channel modulators and modulators of inhibitory GABAergic neurotransmission. However, these drugs are rarely 100% efficacious and their use is often associated with limiting side effects. Thus, there is a clear medical need for novel agents to treat these diseases. One potential mechanism that has not yet been exploited is potassium ($K^+$) channel opening. A significant (and growing) body of genetic, molecular, physiological and pharmacological evidence now exists to indicate that KCNQ-based currents represent particularly interesting targets for the treatment of diseases such as epilepsy and neuropathic pain. Evidence supporting these $K^+$ channels as novel drug targets will be reviewed in the following article. Worldwide patent activity relating to KCNQ channels and KCNQ-modulating drugs and their uses will also be summarised.**

Keywords: epilepsy, KCNQ2, KCNQ3, pain, potassium channel

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## 1. Introduction

Epilepsy and neuropathic pain are disorders characterised by either inappropriate spontaneous neuronal activity or excessive neuronal activity in response to physiological stimuli. These disorders are currently managed by drugs that are capable of dampening neuronal excitability. Drugs approved by regulatory agencies for epilepsy therapy include voltage-gated sodium channel blockers (carbamazepine, phenytoin, lamotrigine and topiramate, for example) voltage-operated calcium channel modulators (ethosuximide, gabapentin and, possibly, levetiracetam) and modulators of inhibitory GABAergic neurotransmission (benzodiazepines, vigabatrin and tiagabine, for example). Of these, gabapentin and carbamazepine are approved for the treatment of neuropathic pain and lamotrigine has demonstrated efficacy for neuropathic pain in clinical trials [1]. Although these drugs provide adequate symptom relief in many patients, a significant number of patients remain poorly treated with the currently available agents. Thus, there is a clear medical need for new drugs with novel mechanisms of action to serve as alternative or adjunct therapy for the treatment of disorders of excessive neuronal excitability.

One potential mechanism that has not yet been exploited is potassium ($K^+$) channel opening. $K^+$ channels play a major role in the control of all aspects of neuronal excitability. Activation of $K^+$ channels theoretically represents a powerful means of reducing excessive neuronal activity.

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## 2. KCNQ channels

KCNQ channels are a family of six transmembrane domain, single pore-loop, voltage-gated $K^+$ channels. Five members of the family have been identified to date, including the cardiac channel KCNQ1 (formerly known as KvLQT1) and four neuronal KCNQ channels, KCNQ2 – 5. In the most recently agreed nomenclature, KCNQ2 – 5 channels have been designated as Kv7.2 – 7.5, respectively [87]. For additional background information, the reader is referred to several excellent recent reviews on the KCNQ family of $K^+$ channels [91,92].

A significant (and growing) body of genetic, molecular, physiological and pharmacological evidence now exists to support the premise that neuronal KCNQ-based currents represent particularly interesting targets for the treatment of diseases such as epilepsy and neuropathic pain. Evidence supporting these $K^+$ channels as novel drug targets will be reviewed in the following article. Worldwide patent activity relating to neuronal KCNQ channels and KCNQ-modulating drugs and their uses will also be summarised.

## 3. KCNQ channels and epilepsy

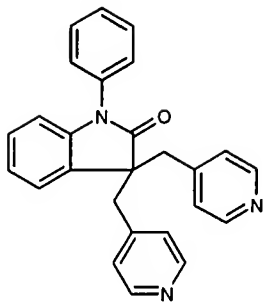
Several lines of evidence suggest that KCNQ-based $K^+$ currents play an important role in the control of CNS excitability and epileptogenesis.

### 3.1 Genetics

The first indication that KCNQ channels may play an important role in the control of CNS excitability came in early 1998, when groups led by Ortrud Steinlein and Mark Leppert independently identified mutations in two novel $K^+$ channel genes, KCNQ2 and 3, as the genetic basis of benign familial neonatal convulsions (BFNC), a rare form of neonatal epilepsy in humans [2-4]. Additional studies have now identified numerous additional mutations, particularly in KCNQ2, which are associated with BFNC [5-12]. Interestingly, the majority of the mutants described to date exhibit loss of function without dominant-negative effects and a mere 25% reduction in KCNQ2 or 3 channel function may be sufficient to cause the electrical instability in BFNC [13]. These observations indicate that KCNQ2 and 3 channels can exert a powerful stabilising influence on CNS excitability. BFNC however, is characterised by seizures in the first few weeks of postnatal life and, in most patients, symptoms spontaneously remit, possibly as a result of the postnatal maturation of the GABAergic inhibitory system and postnatal increases in KCNQ2 expression [14]. The impact of KCNQ2 and 3 mutations on the excitability of the adult human brain is difficult to assess. However, it is known that BFNC patients have an increased risk of developing seizures in later life [85]. This may suggest that the influence of KCNQ2 or 3 mutations on CNS excitability, whilst not as profound as during the neonatal period, carries through into adulthood. The possibility that reductions in KCNQ2 expression could alter CNS excitability during later developmental stages has been addressed using genetically modified mice. Watanabe and colleagues [15] produced mice with targeted deletion of the KCNQ2 gene. Homozygous mice (*Kcnq2*$^{-/-}$) died a few hours after birth as a result of pulmonary atelectasis. Heterozygous mice on the other hand, developed normally and the basal electroencephalograph (EEG) was similar to wild type (*Kcnq2*$^{+/+}$) animals. However, these mice exhibited a hypersensitivity to the convulsant pentylenetetrazol (PTZ). Yang and colleagues [16] have recently identified progeny of ethylnitrosourea (ENU)-treated C57BL/6J mice that exhibit a lowered electroconvulsive threshold. These mice were subsequently shown to harbour a single mutation, Szt1 (seizure threshold 1), consisting of a 300 kb deletion of genomic DNA involving three known genes. Two of these genes, *Kcnq2* and *Chrna4*, are known to be mutated in human epilepsy. Szt1 mice exhibit a phenotype strikingly similar to that described for *Kcnq2* knockout mice. As for *Kcnq2* null mice, homozygous Szt1 mice died shortly after birth as a result of pulmonary atelectasis and heterozygous Szt1 mice displayed an increased sensitivity to PTZ. In addition, Szt1 heterozygous mice were shown to possess a reduced electroconvulsive threshold and evidence of hippocampal abnormalities. Collectively, the available data from genetically modified mice suggests that the influence of KCNQ2 on CNS excitability is not restricted to an early developmental stage but instead, modest reductions in KCNQ2 expression appear to influence CNS excitability throughout postnatal life.

### 3.2 Distribution

Analysis of neuronal KCNQ mRNA expression patterns reveals that these channels are expressed predominantly in the CNS. For example, overlapping expression of KCNQ2 and 3 has been observed in several areas known to be important for human epilepsy and the control of neuronal network oscillations and synchronisation, such as the cortex, thalamus and hippocampus [17-21]. KCNQ2 and 3 channels are colocalised postsynaptically on pyramidal and polymorphic neurons of the human hippocampus and cortex, where they presumably regulate excitability by controlling the integration of synaptic inputs. KCNQ2 channels may also be located presynaptically in the human hippocampus, including mossy fibre bundles and the inner dentate molecular layer, suggesting that these channels may play a role in action potential propagation and neurotransmitter release [20]. A detailed analysis revealed extensive expression of KCNQ2 throughout the mouse brain, with intense somatic expression in modulatory cholinergic and dopaminergic neurons of the basal ganglia and in neurons that regulate hippocampal excitability [21]. Recently, Devaux and colleagues [96] have also demonstrated that KCNQ2 channels are expressed in axon initial segments and nodes of Ranvier throughout the central and peripheral nervous system, where they may play a role in the control of axonal excitability. Relatively little KCNQ2 and 3 expression can be detected in peripheral organs and tissues



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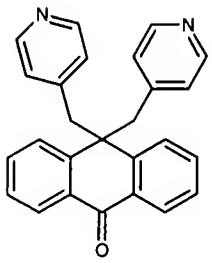

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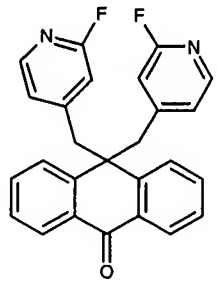

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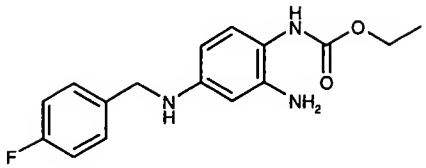
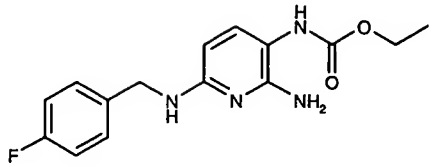


4 Retigabine



5 Flupirtine

[18]. KCNQ5 also shows widespread expression in the CNS, with many regions showing overlapping expression of KCNQ2, 3 and 5 [88,89]. Intense KCNQ5 immunoreactivity was found in both pyramidal and non-pyramidal neurons and a population of glial cells throughout the temporal neocortex and the hippocampal formation of the human brain [94]. KCNQ4 on the other hand, shows restricted expression, being expressed predominantly in the outer hair cells and the nuclei of the central auditory pathway [90].

## 3.3 Function

The overlapping expression of KCNQ2, 3 and 5 in many brain regions suggests that these channels might normally assemble as heteromultimeric channel complexes. Indeed, coexpression of KCNQ2 and 3 in *Xenopus* oocytes leads to the generation of currents at least tenfold larger than those generated by the expression of either channel alone [18]. Furthermore, these currents exhibit very similar biophysical and pharmacological properties to a native neuronal current known to play a powerful role in the control of CNS excitability, the neuronal M-current ($I_M$) [19]. $I_M$ is a non-inactivating, slowly deactivating, subthreshold current first described in sympathetic ganglia and subsequently in a variety of central neurons [19,22-25]. $I_M$ plays an important role in controlling resting membrane potential, responsiveness to synaptic inputs and spike frequency adaptation. $I_M$ is also regulated by neurotransmitters and neuropeptides and $I_M$ inhibition, at least in part, mediates muscarinic and peptidergic excitation of central neurons [86]. KCNQ5 and 3 also form functional heteromeric channels in *Xenopus* oocytes [88,89], suggesting that heterotetramers of KCNQ5 and 3 may also make a significant contribution to the $I_M$ in some brain regions [91]. The finding that heteromeric KCNQ channels represent the molecular correlate of $I_M$, strongly supports a role for these channels in the control of CNS excitability. These findings also suggest that KCNQ channels, especially as heteromeric complexes, represent promising targets for drugs designed to alter neuronal excitability.

## 3.4 Pharmacology

### *3.4.1 Pharmacological agents for the study of KCNQ channels*

A number of pharmacological tools have been identified which modulate KCNQ and/or M-currents. The first selective $I_M$ inhibitor described was linopirdine (compound 1). This compound blocks neuronal M-currents at concentrations that are without effect on other neuronal $K^+$ currents [26-28]. Linopirdine also blocks KCNQ channels at concentrations that are without effect on related $K^+$ channels [19,93]. Related compounds include XE-991 (compound 2) and DMP-543 (compound 3) [29,30].

Retigabine (compound 4, N-(2-amino-4-[fluorobenzylamino]-phenyl) carbamic acid, D-23129) and flupirtine (compound 5) are KCNQ and $I_M$ activators. Retigabine was first identified as a $K^+$ channel opener by Chris Rundfeldt [31,32], who showed that this drug could increase barium and weakly tetraethylammonium sensitive, 4-aminopyridine-insensitive $K^+$ conductance in NG108-15, hNT, PC12 cells and isolated mouse cortical neurons. In addition, retigabine has been shown to induce membrane hyperpolarisation in neurons in rat hippocampal–entorhinal cortex slices [33]. Until recently, the molecular nature of the $K^+$ channel opened by retigabine was unknown. In 2000 however, three groups independently identified KCNQ2/3 channels as a molecular target for retigabine [34-36]. Data generated by these groups demonstrated that retigabine, at concentrations between 0.1 and 10 μM, enhanced KCNQ2/3 currents by inducing profound leftward shifts in the voltage-dependence

of channel activation. Additional studies have demonstrated that retigabine appears to be a relatively non-selective KCNQ channel modulator, being capable of activating all members of the KCNQ channel family except KCNQ1 [37,93]. Retigabine also enhances native M-currents in PC-12 cells, rat sympathetic neurons and rat dorsal root ganglion cells [36-38]. Evidence also exists to suggest that retigabine may possess additional actions. For example, retigabine has been shown to increase the synthesis of GABA in rat hippocampal slices and to enhance GABA-induced chloride currents in cultured rat cortical neurons [39,40]. Retigabine may also possess weak sodium and calcium channel blocking activity [40]. However, these effects are generally only seen at concentrations 10 – 100-fold higher than those required for KCNQ activation [41]. It seems therefore likely, that KCNQ activation represents the primary pharmacological action of retigabine.

In addition to retigabine, recent preliminary data suggests that a structurally related compound, flupirtine, also activates KCNQ2/3 channels and native M-currents. Although flupirtine is generally considered to be either an NMDA antagonist or an $\alpha_2$-adrenergic agonist, this is based only on indirect *in vitro* and *in vivo* data. Direct evidence for these mechanisms is noticeably lacking. On the contrary, direct evidence has now emerged to suggest that flupirtine, like retigabine, possesses the ability to enhance the activation of KCNQ2/3 $K^+$ channels. Using whole-cell patch clamp recordings from HEK293 cells transiently transfected with KCNQ2/3 constructs, Ilyin and colleagues [42] determined that flupirtine (10 µM) was a positive modulator of KCNQ channels with a mechanism of action similar to that of retigabine. Flupirtine increased current amplitude, caused a hyperpolarising shift in the KCNQ2/3 activation curve and slowed KCNQ2/3 deactivation kinetics.

### 3.4.2 *KCNQ modulators in* in vitro *and* in vivo *models of epilepsy*

The agents described in the preceding section (linopirdine, XE-991, retigabine and flupirtine) have been used to help define the role of KCNQ channels and M-currents in the control of CNS excitability. KCNQ blockers, such as linopirdine and XE-991, appear to increase neuronal excitability as evidenced by their ability to increase neurotransmitter release and enhance cognition in a variety of animal models [43-45]. Furthermore, the KCNQ $K^+$ channel inhibitor linopirdine (30 mg/kg) is proconvulsant [46].

Retigabine and, to a lesser extent, flupirtine have been evaluated in animal models of epilepsy. Consistent with the widespread distribution of KCNQ channels and the important role played by these channels, retigabine exerts anticonvulsant activity in a broad range of seizure models. Retigabine prevents epileptiform activity induced by 4-aminopyridine, bicuculline, low magensium ($Mg^{2+}$) and low calcium ($Ca^{2+}$) in hippocampal slices [47,48] and seizures induced by PTZ, maximal electroshock, kainate, penicillin, picrotoxin and NMDA in rodents [49-51]. Retigabine is also effective against audiogenic seizures in DBA/2J mice, against seizures in epilepsy-prone rats and against seizures in an amygdala-kindling model [51-53]. Flupirtine is effective against PTZ-induced seizures in mice [54].

### 3.5 Clinical

Further support for the validity of KCNQ channels as targets for novel antiepileptic agents comes from clinical studies with retigabine and flupirtine. Retigabine is being developed for the treatment of epilepsy and has been evaluated in five Phase IIa (efficacy and dose-range-finding) clinical trials as well as a long-term extension study [55]. In two add-on, open-label, studies in patients with treatment-resistant partial seizures (> 4 seizures/month), 12 out of 35 patients completing the studies showed > 50% reduction in seizure frequency [56]. In a larger, randomised, double-blind, placebo-controlled dose-ranging add-on study in 399 patients, retigabine at 900 and 1200 mg resulted in statistically significant reductions in seizures. Median reductions of 13, 23, 29 and 35% for placebo and daily doses of 600, 900 and 1200 mg, respectively, were observed. It was found that 16, 23, 32 and 33% of patients exhibited a 50% or greater reduction in seizures (ie. the responder population) following daily doses of placebo, 600, 900 and 1200 mg, respectively [55]. In pharmocokinetic studies, plasma concentrations reached ~ 5 µM at a minimally effective dose of retigabine (350 mg b.i.d.) [57]. These concentrations are very similar to those required for KCNQ channel opening, supporting the involvement of these channels in the anticonvulsant effects of retigabine.

Flupirtine has been evaluated in a small-scale clinical trial involving 4 patients with refractory epilepsy. Flupirtine (400 mg/day) was administered in conjunction with existing antiepileptic therapy and all 4 patients showed a decrease in seizure frequency [54]. In a second trial, 400 – 800 mg/day flupirtine, administered with existing therapy, reduced seizure frequency in eight out of nine patients [58].

In addition to providing information on the possibility that activation of KCNQ channels can be beneficial for the treatment of epilepsy, clinical studies can provide information regarding unwanted adverse effects associated with KCNQ activation. In healthy volunteers administered increasing doses of retigabine, dizziness was the most frequent adverse event and occurred in a dose-related manner [57]. Dizziness was not reported by any subjects receiving placebo. Dose-limiting adverse events observed at 600 mg retigabine were chills, pain, symptomatic hypotension, dizziness, nausea, myalgia, sweating and vomiting. Asthenia and somnolence were also reported in the study. In patients administered retigabine as add-on therapy to their existing antiepileptic drugs, the most common adverse events were asthenia, dizziness, headache, somnolence, tremor, speech disorder, amnesia, ataxia, blurred vision, mental slowing and vertigo [55,56].

## 4. KCNQ channels and neuropathic pain

### 4.1 Distribution

Painful stimuli are transferred to the CNS by the lateral spinothalamic tract. First order neurons transmitting pain impulses from the skin (Aδ and C fibres) enter the substantia gelatinosa of the dorsal horn via the dorsal roots. Second order neurons in the lateral spinothalamic tracts convey impulses associated with pain up to the nuclei of the ventroposterior thalamus (ventroposterior medial nucleus [VPM] and ventroposterior lateral nucleus [VPL]) where the painful impulses are integrated. From the thalamus, third order neurons convey the impulses up to the cerebral cortex, where subjective interpretation of pain is thought to occur. Additional background information on pain pathways and processing can be found in an excellent review article by Hunt and Mantyh [98]. Additional information concerning pain terminology can be found at http://www.iasp-pain.org/pubsopen.html [201].

KCNQ2, 3 and 5 mRNA is expressed at key locations in the pain pathway. Expression of KCNQ2 and 3 has been identified in the thalamus, including the ventroposterior thalamus, the cerebral cortex and the dorsal and ventral spinal cord [17-19,21,97]. In the periphery, KCNQ2, 3 and 5 mRNA can be found in first order sensory neurons in the isolated rat dorsal root ganglia [38,59]. In addition, using immunofluorescence and electrophysiological techniques, David Brown's group has shown that KCNQ2, 3 and 5 protein and M-currents are expressed in cells of the rat DRG. Immunofluorescence studies using selective antibodies identified expression of these subunits in the somata and neuronal processes of both small and large diameter DRG neurons. Many cells coexpressed more than one KCNQ subunit. Electrophysiological experiments revealed the presence of linopirdine and retigabine-sensitive M-currents in small and large diameter DRG neurons and many of the small cells that possessed M-currents also responded to capsaicin, identifying them as nociceptive neurons [38]. In addition, as noted in Section 3.2, KCNQ2 channels are expressed in axon initial segments and nodes of Ranvier in the peripheral nervous system [96]. The finding that KCNQ channels are expressed in peripheral sensory nerves suggests that, in addition to playing a role in central pain perception, KCNQ channels may also play a role in peripheral pain processing.

### 4.2 Pharmacology

Direct evidence supporting a role for KCNQ channels in the control of sensory nerve excitability and pain processing comes predominantly from pharmacological studies. In electrophysiological studies, the KCNQ activator retigabine, has been shown to hyperpolarise isolated small diameter DRG cells (nociceptive neurons) and increase the firing threshold. This effect could be blocked by the KCNQ blockers linopirdine and XE-991 [38]. Passmore and colleagues [38] also studied the effects of retigabine on electrically and naturally (thermal and mechanical) evoked neuronal responses in dorsal horn neurons of both naive rats and rats that had previously undergone L4 and L5 spinal nerve ligation. Retigabine produced a statistically significant, dose-related inhibition of firing in small-diameter C and Aδ nociceptive neurons in both naive and ligated animals. Interestingly, retigabine also seemed highly effective at inhibiting indices of neuronal hyperexcitability such as postdischarge spikes and 'wind-up' in dorsal horn neurons, possibly indicative of an enhanced role for KCNQ channels in hyperexcitable neurons. Indeed, preliminary observations suggest that KCNQ2, 3 and 5 expression in the dorsal root ganglion appears to be maintained following nerve injury, whereas expression of other $K^+$ channels may be decreased (compare [59] with [60-63]).

Recently, Rivera-Arconada and colleagues have also shown that retigabine could depress responses to activation of nociceptive afferent fibres in a rat hemisected spinal cord preparation, whereas XE-991 showed the opposite effect and reversed effects of retigabine [95]. Interestingly, KCNQ modulators exerted only weak effects on non-nociceptive reflexes [95]. Finally, retigabine has been shown to delay the time to peak and reduce the amplitude of the compound action potential in the rat sciatic nerve in a linopirdine-sensitive manner [96].

Collectively, the observations described above clearly support the contention that KCNQ channels and corresponding M-currents may represent novel targets for the treatment of pain. In particular, these channels may represent targets for the treatment of pain states associated with hyperexcitability of peripheral sensory nerves, such as neuropathic and inflammatory pain.

Behavioural studies have also provided evidence to support a role for KCNQ channels in pain perception. Blackburn-Munro and Jensen [64] tested retigabine in several rat models of nociceptive, persistant and chronic pain. In the chronic constriction injury model of neuropathic pain, retigabine attenuated mechanical hypersensitivity to pin prick stimulation and cold allodynia but had no effect on tactile allodynia when measured using von Frey hairs. Attenuation of hyperalgesia to a pin-prick response was also observed with retigabine in the spared nerve model of neuropathic pain but again this compound produced no antiallodynic effect when measured using von Frey hairs. In the formalin model of persistant pain, retigabine attenuated Phase II flinching (a response thought to result from secondary spinal sensitisation) and this effect could be completely reversed by the KCNQ channel blocker XE-991. Acute pain, as measured by Phase I flinching in the formalin test and in the tail flick model, was unaffected by retigabine or XE-991. Importantly, retigabine did not appear to impair motor coordination at the doses tested in the pain models described above. The attenuation of behavioural responses to painful stimuli therefore appears to represent a genuine antinociceptive property of retigabine.

Rostock and colleagues [65] tested retigabine against the thermal hyperalgesia produced by either the ligation or transection of the L5 spinal nerve. In both models, retigabine displayed antihyperalgesic activity as evidenced by

increased paw withdrawal latencies to thermal stimuli. The same authors also tested retigabine in the formalin model and like Blackburn-Munro and Jensen [64], found no activity against Phase I flinching but a significant reduction in Phase II flinching.

In a third study, Passmore and colleagues [38] tested retigabine in the rat carrageenan model of inflammatory pain. Weight distribution was used as a measure of nociception following intraplantar administration of 2% carrageenan. Carrageenan injection produced a significant redistribution of weight bearing such that the inflamed paw bore only 21% of the hind paw load (normal weight bearing would be 50% on each hind paw). Administration of retigabine increased the weight born by the inflamed paw up to 41%, an effect that was blocked by coadministration of XE-991.

As noted above, flupirtine, a close analogue of retigabine also modulates KCNQ channels with a similar mechanism of action to that of retigabine [42]. In animal pain models flupirtine is active in the rat tail flick assay [66] and in the spinal nerve ligation model of neuropathic pain [42], providing further support for the role of KCNQ channels in pain processing and perception.

### 4.3 Clinical

The validity of KCNQ channels as targets for novel analgesic agents is supported by extensive clinical experience with flupirtine. Flupirtine has been marketed in Europe since 1984 as an analgesic under the trade name Katadolon® (Asta Medica). Flupirtine has been shown to be effective for the relief of many types of pain. In double-blind, placebo- or active-controlled studies, flupirtine was efficacious for the treatment of tumour or cancer pain [67-69], for postoperative pain [70-73], for pain following episiotomy [72] and for trauma pain [72]. Doses varied between 100 mg and 300 mg per single dose with administration of up to six doses (usually three) per day. Most studies were conducted using oral administration, but flupirtine was occasionally administered as a suppository.

Flupirtine was effective after a single dose [72] and was effective for up to 1 year of treatment with no evidence of development of tolerance [74]. For tumour/cancer pain, flupirtine efficacy was superior to tramadol, an analgesic with opiate and monaminergic mechanisms of action [75] and pentazocine [69]. In studies of postoperative pain, flupirtine was as effective as pentazocine and dihydrocodeine as well as metamizole, paracetamol and naproxen [71], it was also superior to placebo (69% pain reduction versus 26%) [72]. For postepisiotomy pain, flupirtine was superior to suprofen and for post-traumatic sports injury pain it was superior to paracetamol plus massage. In one controlled trial in migraine patients, there was a trend for flupirtine efficacy [76].

Flupirtine administration was not associated with a high incidence of adverse effects in clinical trials and had fewer adverse effects than the active control drugs, pentazocine and dihydrocodeine [71]. The most common adverse events associated with flupirtine administration in patients were drowsiness, dizziness, dry mouth, pruritis and nausea [72,74,77].

In summary, flupirtine alleviates pain following acute administration without development of tolerance in a variety of painful conditions, including severe pain (cancer and postoperative pain). Low micromolar plasma concentrations (2.5 – 6.5 µM) of flupirtine were associated with doses providing analgesic activity in humans [78]. The only pharmacological activity known to occur in this concentration range for flupirtine is activation of $K^+$ channels [42,79,80]. Thus, while the definitive mechanism of action for flupirtine remains to be resolved, activation of KCNQ channels seems a reasonable hypothesis.

## 5. Summary of patent activity

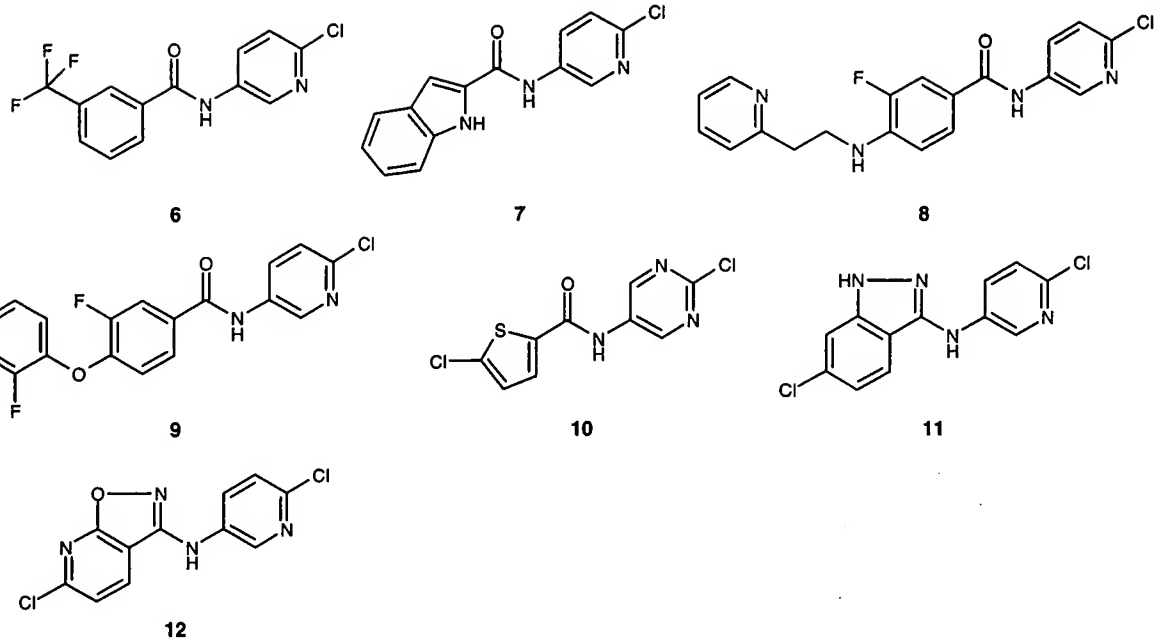
### 5.1 Patents relating KCNQ2, KCNQ3 and KCNQ5 channels

At the time of writing, three US patents had been issued covering KCNQ2 and/or KCNQ3 and uses thereof. In US6403360, Bristol-Myers Squibb (BMS) describes the nucleic and amino acid sequence of human, rat and mouse KCNQ2 and human KCNQ3 and the distribution of these genes in tissues, regions of the brain and the spinal cord. US6403360 also describes the function and preliminary pharmacology of human, rat and mouse KCNQ2 and human KCNQ3 when expressed in *Xenopus* oocytes [101]. In the second patent issued (US6413719), University of Utah Research Foundation describes human KCNQ2 and 3 genes, the nucleic and amino acid sequences of human and mouse KCNQ2 and 3 and the identification of KCNQ2 and 3 mutations in patients with benign familial neonatal convulsions [102]. Finally, in US6472165, Arzneimittelwerk Dresden GmbH present data showing that retigabine is a selective KCNQ2 and 3 activator and describe possible methods for identifying compounds that modulate KCNQ2 or KCNQ2/3 that involve expressing these channel subunits in a cell and either comparing the effect of a substance to that of retigabine or by determining whether a substance is capable of competing with retigabine [103].

Two additional US patents have been issued covering KCNQ5 genes. US6617131 (Aventis Pharma Deutschland GmbH) describes the nucleic and amino acid sequence of human KCNQ5, its distribution in a variety of human tissues and a preliminary evaluation of the function and pharmacology of this gene [123]. US6649371 (Neurosearch A/S) also describes the function and pharmacology of human KCNQ5 [124].

### 5.2 Patents relating KCNQ activation to potential therapeutic targets

The potential role of KCNQ channel activators in reducing neuronal excitability in tissues expressing these channels has led to a plethora of applications and patents covering their use for a wide range of diseases and conditions. Icagen was the first to address the potential of KCNQ activators to effectively modulate pain states and was granted a patent outlining a method for reducing pain in a subject by increasing ion flow



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through KCNQ $K^+$ channels. Selective KCNQ activators were shown to produce dose-dependent analgesic effects in the mouse formalin model [104]. In 2002, BMS published an application describing the use of activators of CNS-associated KCNQ channels for the treatment of migraine or migraine-related disorders [105]. Supporting data focused on the ability of KCNQ compounds from three different series (given i.v.) to reduce superior sagital sinus-stimulated trigeminal field potentials. American Home Products (Wyeth) was recently granted a patent that described KCNQ activators for the use of maintaining bladder control and/or treatment of urinary incontinence [106]. In this patent, KCNQ channels were shown to be expressed in the bladder. Furthermore, retigabine was shown to elicit a concentration-dependent inhibition of KCl-stimulated contractions of isolated rat bladder strips and completely inhibited acetic acid-induced micturition in rats after a 10 mg/kg intraperitoneal dose. Wyeth published another application that outlined the use of KCNQ activators for the treatment or inhibition of hyperactive gastric motility. This claim was supported by the finding that retigabine produced a concentration-dependent inhibition of both KCl- and carbachol-induced ileal contractions [107]. Further experiments showed that the KCNQ blocker XE-991 reversed the retigabine effect.

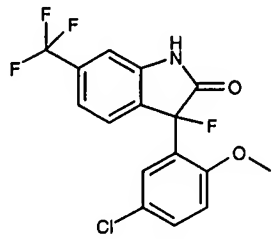
### 5.3 Patents claiming the use of retigabine for non-epileptic conditions

Retigabine (compound 4), a 1,2,4-triaminosubsituted-benzene compound, was found to be an effective anticonvulsant via a traditional *in vivo* screening campaign. Its activity is now believed to be due, in part, to its ability to open KCNQ channels [34-36]. More recently, the closely related pyridine analogue, flupirtine (compound 5), has also been shown to be an effective KCNQ2/3 agonist [42]. The potential use of retigabine (compound 4) for the treatment of a wide variety of disorders related to neuronal excitability has been the basis of several utility patents and patent applications. Wyeth were recently granted a patent which includes the use of retigabine and close analogues for the treatment of anxiety, while Asta Medica have patents claiming the use of retigabine for the treatment of neuropathic pain (retigabine produced a dose-dependent reduction in formalin-induced hyperalgesia), neurodegenerative disorders and for the treatment of chronic reduced cerebral blood supply (retigabine was shown to increase learning ability after constriction of cerebral blood supply in rats) [108-111]. The Glaxo group has an application describing the use of retigabine for the treatment of a number of disorders including cognitive disorders, cancerous diseases, inflammatory processes and ophthalmic diseases, however, no supporting data were reported [112].

### 5.4 Patents describing new KCNQ activators

Since the identification of the KCNQ channel family and their potential therapeutic utility, several programmes to identify selective KCNQ channel agonists using rational drug design techniques have been initiated. During the past 4 years, BMS, Neurosearch and Icagen have disclosed compounds that activate KCNQ channels.

The first series of agonists to be published was a collection of unique 6-substituted-pyridin-3-yl-based benzamides and related heterocyclic amides from Icagen, compounds 6 and 7 being representative examples [104,113,114]. Further explorations around the phenyl moiety of the benzamide compounds led to the identification of numerous additional analogues, such as compounds 8 and 9 [115]. A subsequent patent application discloses 2-substituted-pyrimidin-5-yl compounds (such as compound 10) [116]. The utilisation of several different conformational constraints/amide bond bioisosteres to the Icagen benzamides generated



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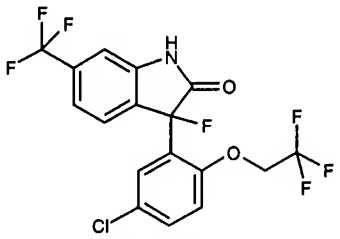

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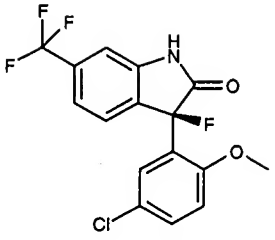
15 (+) Enantiomer



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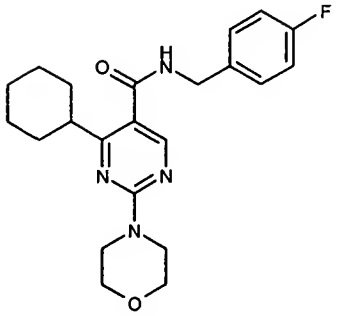

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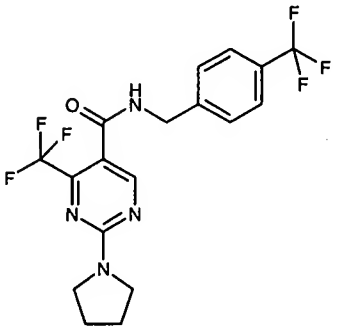

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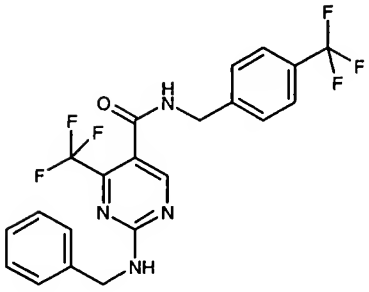

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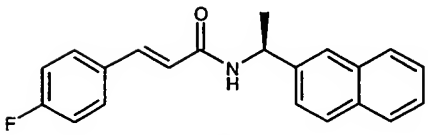

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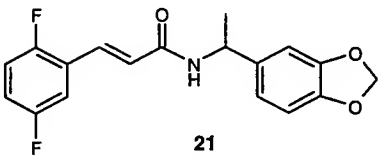

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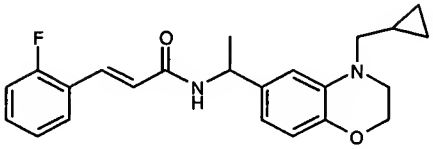

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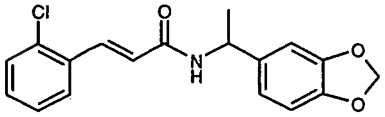

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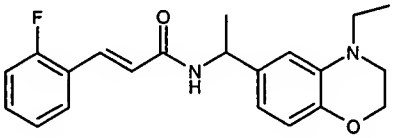

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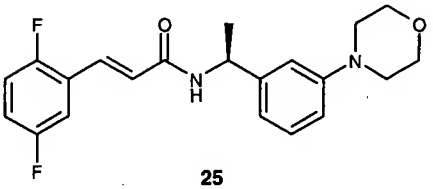

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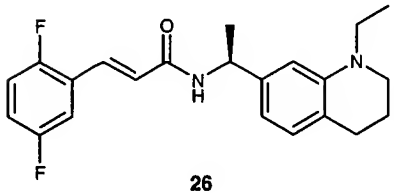

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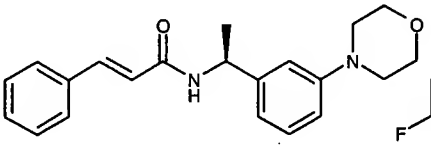

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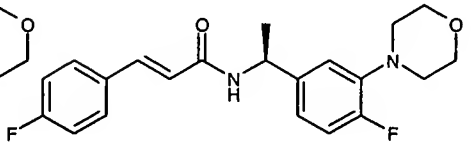

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bicyclic KCNQ agonists, such as the indazoles (compound 11) and benzisoxazoles (compound 12) [117].

Scientists at Neurosearch identified the racemic fluorooxindole (compound 13) as an agonist of KCNQ family members KCNQ4 (effective concentration for half-maximum response [$EC_{50}$] value = 2.4 μM) and KCNQ5 and filed an application regarding the use of this chemical class for a variety of conditions [81,122]. Both the racemate (compound 14) and its (+)-enantiomer (compound 15) were found to increase current flow through murine KCNQ2 channels at 10 μM [82,118,119]. Interestingly, the (+)-enantiomer of compound 13, compound 16, has already been in clinical trials based upon its maxi-K activity.

BMS have reported two other classes of small molecule agonists during the last 2 years. The 2,4-disubstituted pyrimidine-5-carboxamide agonists are exemplified by compounds 17 – 19 [120]. The trifluoromethoxy compound 18 increased current flow through murine KCNQ2 channels when tested

at 5 μM. Compound 18 also caused a dose-dependent reduction in superior sagital sinus stimulated trigeminal field response model of migraine. The second series of compounds were the cinnamide derivatives [121]. Compounds 20 – 22 possessed $EC_{50}$ values against murine KCNQ2 channels in the micromolar range, whereas the structurally similar compounds 23 and 24 were considerably more potent with $EC_{50}$ values of 0.6 and 0.9 nM, respectively. A comparison of compounds 21, 25 and 26 revealed that structurally and electronically different aryl systems did not significantly affect potency. Several different electron-modulating groups were tolerated on the cinnamide aryl. Small halogens, such as fluoro and chloro, provided good activity and presumably greater *in vivo* stability. Interestingly, the switch between 2,5-difluorophenyl (compound 21) and 2-chlorophenyl (compound 23) resulted in an almost 1000-fold increase in potency.

A number of the BMS compounds were tested in animal models of migraine, anxiety, neuropathic pain and others. When given intravenously (typically 1 mg/kg) the test compounds caused a reduction in the number of spreading depressions comparable to the effect observed with 100 mg/kg of intravenous valproic acid in a rat cortical spreading depression model. Compounds were also shown to be reasonably efficacious in rat models of neuropathic pain (Chung and Streptozotocin models), however higher doses were generally required (typically 10 mg/kg, i.v.). More information regarding the *in vitro* and *in vivo* development of this chemical class of compounds has been recently published. Compound 27 was found to open murine KCNQ2 channels in single cell voltage-clamp experiments, with an $EC_{50}$ value of 3.3 μM and it hyperpolarised the membrane potential in SH-SY5Y human neuroblastoma cells expressing endogenous KCNQ channels, with an $EC_{50}$ value of 0.69 μM. Compound 27 also exhibited good oral bioavailability in both rats and dogs and doses of 30 and 10 mg/kg (intragastric) of compound 26 were found to be efficacious in a rat model of migraine [83]. Unfortunately compound 27 suffered from undesired CYP450 interactions. The selectively fluorinated analogue 28 however, maintained good potency against the murine and endogenously expressed human KCNQ2 channels ($EC_{50}$ values of 1.2 and 1.55 μM, respectively) and was devoid of these unwanted cytochrome P450 interactions [84].

## 6. Expert opinion

The findings described above provide a high level of validation for KCNQ channels as antiepileptic and analgesic drug targets. However, it should be noted that pharmacological studies with retigabine and flupirtine are central to the argument that KCNQ channels represent valid therapeutic drug targets and, as noted above, these drugs may possess actions unrelated to KCNQ opening. For example, retigabine has been shown to enhance GABAergic transmission in the CNS. Similarly, it has been proposed that flupirtine may interact with NMDA and α2-adrenergic receptors. It is unclear, therefore, if the efficacy of retigabine and flupirtine in animals models of epilepsy and pain and in human studies is entirely due to KCNQ activation. Final validation of KCNQ channels as antiepileptic and analgesic drug targets therefore, will require the identification of novel, highly selective KCNQ openers. *In vivo* data with novel, selective KCNQ activators is eagerly awaited and should provide the final piece of evidence to unequivocally validate KCNQ as therapeutic drug targets for the treatment of epilepsy and pain.

## Disclaimer

The opinions expressed in this paper are solely those of the authors and are not to be attributed to Icagen, Inc.

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## Website

201. http://www.iasp-pain.org/pubsopen.html International Association for the Study of Pain website, publications page


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